PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/18, C12M 1/20 | A1

(11) International Publication Number: WO 94/16097

(43) International Publication Date: 21 July 1994 (21.07.94)

(21) International Application Number: PCT/IT94/00001

(22) International Filing Date: 3 January 1994 (03.01.94)

(30) Priority Data:
RM93A000002 4 January 1993 (04.01.93) IT

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIBIOTIC ASSAY AND KITS FOR THE USE THEREOF

(57) Abstract

A method to assay a microorganism growth, or a microorganism growth inhibition, in the presence of an effective antibiotic amount, as a function of the pH changes in the culture medium and a color change of a color indicator, is described. The method and the kit therefom are to be used to assay samples from biological fluids, water, effluents, etc.

higher precision. In all of these systems the bacterial antibiotic resistance is assayed by means of bacterium growth in a liquid medium in the presence of the antibiotic; being the bacterial growth detection evidetiated by means of a turbidity reading, either by sigth or automatic spectrophotometers. The kit derived therefrom is available on the market from Bio-Mérieux, France.

This assay type shows some advantages, when compared with the conventional Kirby and Bauer's method; but it is also involving art-skilled operators and expensive equipment; furthermore the method does not allow a clear and univocal result evaluation, as it often shows intermediate values.

The authors of the present invention developed a system to detect bacterial growth, or an inhibition of bacterial growth, in the presence of an effective amount of antibiotics, as function of pH changes in the culture medium. "Effective amount" here means a concentration, that is able to inhibit the growth of a microorganism strain, which is sensitive to such given antibiotic. pH changes, depending from the metabolism of sugars into the culture medium, are detected by means of color indicators. Results fully agree with the previously described methods and, in some cases, shows even a higher sensitivity. Further, the method is easy to apply, not expensive and no skilled operators are requested. Finally, the result readings are clear and univocal.

It is an object of the present invention a method to detect the microorganism growth from a sample into a liquid culture medium in the presence of an effective amount of an antibiotics, wherein said microorganism growth is detected by identifying pH changes in said culture medium, so that the pH becomes more acidic in respect of the pH of the culture medium,

when no growth occurs. Preferably, pH changes are identified by a pH color indicator contained into said culture medium.

It is a further object of the invention a method to detect the microorganism growth from a sample, comprising the steps, as follows:

- to add a microorganism suspension to a culture medium suitable to grow said microorganism and containing a pH color indicator, preferably phenolsulfonphtalein (red phenol), to get a mixture;
- to load sterile containers with fractions of said mixture, each container containing an effective amount of an antibiotics;
- to incubate said mixture at a growth permissive temperature for said microorganism for at least 16 hours, preferably from 18 up to 24 hours; and
- to verify the color of said culture medium.

Preferably, said culture medium for said microorganism comprises:

- Müller-Hinton's broth	21	g/l
- K_2HPO_4	0.3	g/l
- Glucose	30	g/l
- $MgCl_2$	0.25	g/l
- $CaCl_2$	6.7	g/l
- Red phenol (0.3 %) in H_2O	42	ml/l
- Horse serum	10	ml/l

being the pH in the range of 7.0 - 7.4.

Said containers may comprise test tubes, trays, microtitration plates, wherein the antibiotic was previously added steadily. For convenience, microtitration trays comprise different wells, each one containing a different amount of different antibiotics, in order to enable to assay various antibiotics at the same time.

It is a further object of the invention a kit to detect growth a microorganism from a sample in the

presence of an effective amount of an antibiotics, comprising:

- a sterile microtitration plate, comprising different wells, each one containing an antibiotic effective amount in a stabilized state; said plate at least containing two different antibiotics in two different wells and at least one antibiotic-free well;
- a sterile container containing an adequate amount of a culture medium suitable to promote the growth of said microorganism as well as a pH indicator, preferably a red phenol.

According to a preferred embodiment of the invention, said antibiotic is contained at least into a pair of wells, at the minimum effective concentration in the former and at a higher concentration in the latter.

According to the invention, the plate is prepared by dissolving the antibiotic as instructed by the Supplier, diluting properly the obtained solution, filling wells at the same time with 50 µl of said solution, by using an automatic multichannel dosing device, and finally dehydrating said wells until the solvent is full evaporated, at a temperature not able to deactivate said antibiotics.

Sterile plates could be maintained up to one year at a temperature ranging from 4°C up to 8°C.

The invention shall be described in the following by reference to some explicative, but not limiting, examples, which are related to different samples as well as to some comparison tables in respect of other assay tecniques.

Example 1 QUICK ANTIBIOTIC ASSAY ON URINE GERMS

The system consists of 17 dried antibiotics into various wells of a microtitration tray at single specific concentrations, allowing to assay the sensitivity to antibiotics of the most common bacteria,

that could be collected from an urine culture. Bacteria are resuspended in a culture medium, containing a growth indicator, and then loaded in each system wells. After having been incubated 18-24 hours at 37°C, the automated or displayed reaction readings are carried out.

Procedure:

1) to take a plate;

2) to obtain a bacterial suspension with an opacity corresponding to 0.5 of the McFarland's standard;

3) to transfer the following amounts of this suspension to the test tube, containing the culture broth with the red phenol:

a) 10 µl of Gram negative bacteria;

b) 200 µl of Gram positive bacteria;

4) to load each well with 0.2 ml of the bacterial suspension;

5) to put over the well a proper cover after having recorded the patient's name, the assay date and the bacterium type;

6) to incubate for 18-24 hours at 37°C;

7) to check the color change in the control well (bacterium growth well) and evaluate the results.

Antibiotics are listed in Table 1.

TABLE 1

Antibiotics contained in the system

WELL Nr.	CODE	ANTIBIOTIC	mg/l
1	F	Nitrofurantoin	100
2	NA	Nalidixic acid	16
3	NOR	Norfloxacillin	8
4	CIP	Cyprofloxacillin	2
5	PEF	Pefloxacillin	4
6	KF	Chefalotin	32
7	CFD	Cefonicid	32
8	CAZ	Ceftadizime	32

9	CN	Gentamicin	8
10	TOB	Tobramycin	8
11	AK	Amikacin	16
12	SXT	Co-trimoxazole	8
13	ATM	Aztreonam	32
14	AMP	Ampicillin	16
15	AMX	Amoxycillin	16
16	MEZ	Mezlocillin	32
17	PRL	Piperacillin	64
18	C	Control	

Readings and evaluation:

Red to yellow color changes are recorded for each well and results are evaluated according to the following table 2:

TABLE 2

COLOR	BACTERIAL GROWTH	THE BACTERIUM IS:
Red	-	S = sensitive
Orange	+/-	I = partial sensitive
Yellow	+	R = resistant

Some no-glucose fermenting and oxidase negative bacteria cause no yellow shifts of red phenol, used as bacterial growth indicator; therefore the antibiotic-resistance is visualized by well turbidity as follows:
CLEAR RED = S = SENSITIVE; and
TURBID RED = R = RESISTANT

After the use, plates, test tubes and pipets may be decontaminated by a sodium hypochlorite incubation, incinerated or processed on autoclave.

In order to check the method standardization, control strains are used from ATCC, Maryland, US.

The kit may be stored for one year thereafter at a temperature ranging from 2°C up to 8°C.

Example 2 TWO CONCENTRATION ANTIBIOTIC ASSAY ON URINE GERMS

The system consistS of 12 dual concentrated, dried antibiotics. The assay procedure is as in Example 1, but the antibiotics are listed in table 3:

TABLE 3

Antibiotics contained in the system

WELL NR.	CODE	ANTIBIOTIC	µg/ml C	µg/ml C
1-2	F	Nitrofurantoin	25	100
3-4	NA	Nalidixic acid	8	16
5-6	NOR	Norfloxacillin	1	8
7-8	AMX	Amoxycillin	4	16
9-10	PRL	Piperacillin	16	64
11-12	KF	Chefalotin	8	32
13-14	CFD	Cefonicid	4	32
15-16	CAZ	Ceftadizime	4	32
17-18	CN	Gentamicin	4	8
19-20	TOB	Tobramicin	4	8
21-22	AK	Amikacin	8	16
23	SXT	Co-trimoxazole	8	
24	C	Control		

Red to yellow shifts are recorded for each well contents and the results evaluated as in Table 4:

TABLE 4

COLORS OF THE TWO WELLS (each antibiotic)	BACTERIAL GROWTH C	C	THE BACTERIUM IS
Red/red	-	-	S = sensitive
Orange/red	+/-	-	MS = mid sensitive
Yellow/red	+	-	LS = lightsensitive
Yellow/orange	+	+/-	MR = mid resistant
Yellow/yellow	+	+	R = resistant

Red/yellow	-	+	Test not in conformity (not valid)

wherein: c = lower antibiotic concentration;
C = higher antibiotic concentration;
- = no bacterial growth (red);
+ = bacterial growth (yellow); and
-/+ = small bacterial growth (orange).

Example 3 QUICK ASSAY ON NEGATIVE BACTERIA

The system consists of 12 dual concentrated, dried antibiotics, and allows to assay the sensitivity of the more common Gram negative bacteria (Negative Oxidase) to antibiotics. The procedures are the same described in the Example 1, but the concerned antibiotics are as listed in Table 5 herebelow.

TABLE 5

Antibiotics contained in the system

WELL NR.	CODE	ANTIBIOTIC	µg/ml c	µg/ml C
1-2	AMP	Ampicillin	4	16
3-4	MEZ	Mezlocillin	8	32
5-6	KF	Chefalotin	8	32
7-8	CAZ	Ceftadizime	4	32
9-10	CFD	Cefonicid	4	32
11-12	CN	Gentamicin	4	8
13-14	TOB	Tobramicin	4	8
15-16	AK	Amikacin	8	16
17-18	TET	Tetracycline	4	8
19-20	C	Cloramphenicol	8	16
21-22	CIP	Cyprofloxacin	1	2
23	SXT	Co-trimoxazole	8	
24	C	Control		

The red to yellow shifts are recorded for each well contents and the results evaluated according to the criteria as described in the Example 2.

Example 4: STAPHYLOCOCCUS ASSAY

The system consists of dual concentration dried antibiotics, and allows to carry out the antibiotic assay for the staphylococcus bacteria. The procedure is the same as described in the Example 1, but the antibiotics are used as listed in the Table 6 herebelow:

TABLE 6

Antibiotics contained in the system

WELL NR.	CODE	ANTIBIOTIC	µg/ml C	µg/ml C
1-2	ERY	Erythromycin	1	4
3-4	PEF	Pefloxacin	1	4
5-6	CIP	Cyprofloxacillin	1	2
7-8	SXT	Co-trimoxazole	2	8
9-10	CFD	Cefonicid	4	32
11-12	TEC	Teichoplanin	4	16
13-14	CN	Gentamicin	4	8
15-16	AK	Amikacin	8	16
17-18	FOS	Phosphomycin	32	64
19-20	PRL	Piperacillin	16	64
21-22	AMS	Ampicillin/Sulbactam	8/4	16/8
23	OXA	Oxacillin	2	
24		Control		

The red to yellow shifts are recorded for each well contents and the results evaluated according to the the criteria as described in Example 2.

Example 5: STREPTOCOCCUS ASSAY

The system contains 12 dual concentrated dried antibiotics, and allows to carry out the Streptococcus assay. The same procedure of the Exaple 1 is followed, but the system contains the antibiotic as listed in the Table 7 herebelow:

Table 7

Antibiotic contained in the system

WELL NR.	CODE	ANTIBIOTIC	µg/ml C	µg/ml C
1-2	ERY	Erythromycin	1	4
3-4	PEF	Pefloxacillin	1	4
5-6	CIP	Cyprofloxacillin	1	2
7-8	SXT	Co-trimoxazole	2	8
9-10	CFD	Cefonicid	4	32
11-12	CAZ	Ceftadizime	4	32
13-14	TEC	Theicoplanin	4	16
15-16	RD	Rifamycin	4	16
17-18	AMS	Ampicillin/Sulbactam	8/4	16/8
19-20	TCC	Ticarcillin/Clavulanic acid	16/1	64/4
21-22	PRL	Piperacillin	16	64
23	OXA	Oxacillin	2	
24		Control		

The red to yellow shifts are recorded for each well contents and the results evaluated according to the criteria as described in the Example 2.

Example 6: GRAM POSITIVE ASSAY

The system consists of single concentrated dried antibiotics, and allows to assay the sensitivity of the gram positive bacteria to the antibiotics. The procedure followed as in Example 1, however the concerned antibiotics are enlisted as in the Table 8 herebelow.

Table 8

Antibiotics contained in the system

WELL NR.	CODE	ANTIBIOTIC	µg/ml
1	CN	Gentamicin	8
2	AK	Amikacin	16
3	TOB	Tobramycin	8
4	FOS	Phosphomycin	64

5	TEC	Theicoplanin	16
6	TET	Tetracycline	8
7	RD	Rifamycin	16
8	ERY	Erythromycin	4
9	CIP	Cyprofloxacillin	2
10	PEF	Pefloxacillin	4
11	SXT	Co-trimoxazole	8
12	CFD	Cefonicid	32
13	CAZ	Ceftadizime	32
14	AMS	Ampicillin/Sulbactam	16/8
15	TCC	Ticarcillin/Clavulanic acid	64/4
16	PRL	Piperacillin	64
17	OXA	Oxacillin	2
18	C	Control	

The red to yellow shifts are recorded for each well contents and the results evaluated according to the criteria as described in the Example 1.

Example 7: COMPARISON WITH OTHER ASSAY METHODS

The following comparison tables are obtained using certified bacterial strains from American Tissue Culture Collection (ATCC), Maryland, US.

BACT.: E.Coli (ATCC 25922)ATR-1 API-BIOMER. HINTON'S DISK

F	Nitrofuroantoin	100	S	S	S
NA	Nalidixic acid	16	S	S	S
NOR	Norfloxacillin	8	S	S	S
CIP	Cyprofloxacillin	2	S	S	S
PEF	Pefloxacillin	4	S	S	S
KF	Chefalotin	32	S	S	S
CFD	Cefonicid	32	S	S	S
CAZ	Ceftadizime	32	S	S	S
CN	Gentamicin	8	S	S	S
TOB	Tobramicin	8	S	S	S

AK	Amikacin	16	S	S	S
SXT	Co-trimoxazole	8	S	S	S
ATM	Aztreonam	32	S	S	S
AMP	Ampicillin	16	R	R	R
MX	Amoxycillin	16	R	I	R
MEZ	Mezlocillin	32	S	S	S
PRL	Piperacillin	64	S	S	S
C	Control		GROWTH +		

S = sensitive (red)
R = resistant (yellow)
I = intermediate (orange)

BACT.:Streptofecalis(ATCC19433)ATR-1 API-BIOM. HINTON'S DISK

F	Nitrofurantoin	100	S	S	S
NA	Nalidixic acid	16	R	R	R
NOR	Norfloxacillin	8	S	S	S
CIP	Cyprofloxacilin	2	S	S	S
PEF	Pefloxacillin	4	S	S	S
KF	Chefalotin	32	S	S	S
CFD	Cefonicid	32	R	R	R
CAZ	Ceftazidime	32	I	R	R
CN	Gentamicin	8	R	R	R
TOB	Tobramicin	8	S	S	S
AK	Amikacin	16	R	R	R
SXT	Co-trimoxazole	8	S	S	S
ATM	Aztreonam	32	R	R	R
AMP	Ampicillin	16	S	S	S
AMX	Amoxycillin	16	R	R	R
MEZ	Mezlocillin	32	S	S	S
PRL	Piperacillin	64	S	S	S
C	Control		GROWTH +		

	BACT. Providencia Stuartii		ATR-1 API-BIOM.		HINTON'S DISK
F	Nitrofurantoin	100	S	S	S
NA	Nalidixic acid	16	S	S	S
NOR	Norfloxacillin	8	S	S	S
CIP	Cyprofloxacillin	2	S	S	S
PEF	Pefloxacillin	4	S	S	S
KF	Chefalotin	32	R	R	R
CFD	Cefonicid	32	S	S	S
CAZ	Ceftazidime	32	S	S	S
CN	Gentamicin	8	S	S	S
TOB	Tobramicin	8	S	S	S
AK	Amikacin	16	S	S	S
SXT	Co-trimoxazole	8	S	S	S
ATM	Aztreonam	32	S	S	S
AMP	Ampicillin	16	R	R	R
AMX	Amoxycillin	16	R	R	R
MEZ	Mezlocillin	32	S	S	S
PRL	Piperacillin	64	S	S	S
C	Control		GROWTH +		

	BACT. E. Coli			ATR-2 API-BIOM.		HINTON'S DISK
F	Nitrofurantoin	25	100	S	S	S
NA	Nalidixic acid	8	16	S	S	S
NOR	Norfloxacillin	1	8	S	S	S
AMX	Amoxycillin	4	16	I	I	I
PRL	Piperacillin	16	64	S	S	S
KF	Chefalotin	8	32	I	I	I
CFD	Cefonicid	4	32	S	S	S
CAZ	Ceftazidime	4	32	S	S	S
CN	Gentamicin	4	8	S	S	S
TOB	Tobramicin	4	8	S	S	S
AK	Amikacin	8	16	S	S	S
SXT	Co-trimoxazole		8	S	S	S
C	Control			GROWTH +		

				ATR-2 API-BIOM.		HINTON'S DISK
BACT.: E. Cloacae						
F	Nitrofurantoin	25	100	I	I	I
NA	Nalidixic acid	8	16	S	S	S
NOR	Norfloxacillin	1	8	S	S	S
AMX	Amoxycillin	4	16	R	R	R
PRL	Piperacillin	16	64	S	S	S
KF	Chefalotin	8	32	R	R	R
CFD	Cefonicid	4	32	R	R	R
CAZ	Ceftadizime	4	32	S	S	S
CN	Gentami-cin	4	8	S	S	S
TOB	Tobramicin	4	8	S	S	S
AK	Amikacin	8	16	S	S	S
SXT	Co-trimoxazole		8	S	S	S
C	Control			GROWTH +		

				ATR-3 API-BIOM.		HINTON'S DISK
BACT.: Ent. Cloacae						
AMP	Ampicillin	4	16	R	R	R
MEZ	Mezlocillin	8	32	S	S	S
KF	Cefalotin	8	32	R	R	R
CAZ	Ceftazidime	4	32	S	S	S
CN	Gentamicin	4	8	S	S	S
TOB	Tobramicin	4	8	S	S	S
AK	Amikacin	8	16	S	S	S
TET	Tetracycline	4	8	S	S	S
C	Chloramphenicol	8	16	S	S	S
CIP	Cyprofloxacillin	1	2	S	S	S
SXT	Co-trimoxazole	8		S	S	S
C	Control			GROWTH +		

				ATR-4	API-BIOM.	HINTON'S DISK
BACT.: Staphylococcus aureus						
ERY	Erythromycin	1	4	S	S	S
PEF	Pefloxacillin	1	4	S	S	S
CIP	Cyprofloxacillin	1	2	S	S	S

SXT	Co-trimoxazole	2	8	S	S	S
CFD	Cefonicid	4	32	S	S	S
TEC	Theicoplanin	4	16	S	S	S
CN	Gentamicin	4	8	S	S	S
AK	Amikacin	8	16	S	S	S
FOS	Phosphomycin	32	64	I	I	R
PRL	Piperacillin	16	64	S	S	S
AMS	Ampicillin/ Sulbactam	8/4	16/8	S	S	S
OXA	Oxacillin	2		S	S	S
C	Control			GROWTH +		

CLAIMS

1. A method to assay the microorganism growth from a sample in a liquid culture medium in the presence of an effective amount of an antibiotics, wherein said growth is detected by identifying pH changes in said culture medium, so that the pH becomes more acidic in respect of the pH of the culture medium, when no growth occurs.

2. A method to assay the microorganism growth from a sample according to claim 1, wherein said pH changes are detected by means of a pH color indicator contained whitin said culture medium.

3. A method to assay the microorganism growth from a sample according to claim 2, wherein said pH color indicator is red phenol.

4. A method to assay the microorganism growth from a sample according to any of previous claim, comprising the steps as follows:

- to add a microorganism suspension in a culture medium permissive to the growth of said microorganism and containing a pH color indicator, preferably red phenol, to get a mixture;
- to load sterile containers with fractions of said mixture, each container containing an effective amount of an antibiotics;
- to incubate said mixture at a growth permissive temperature for said microorganism for at least 16 hours, preferably from 18 up to 24 hours; and
- to verify the color of said culture medium.

5. Method to assay the microrganism growth from a sample according to the claim 4, wherein said suitable culture medium to promote the growth of said microorganism comprises:

- Müller-Hinton broth	21 g/l
- K_2HPO_4	0.3 g/l
- Glucose	30 g/l

- $MgCl_2$ 0.25 g/l
- $CaCl_2$ 6.7 g/l
- Red phenol (0.3%) in H_2O 42 ml/l
- Horse serum 10 ml/l

ranging the pH of the culture medium from 7.0 to 7.4.

6. A method to assay the microorganism growth from a sample according to any claim 4 or 5, wherein said containers include test tubes, trays, microtitration trays to which the antibiotic was previously added steadily.

7. A method to assay the microorganism growth from a sample according to the claim 6, wherein said microtitration trays comprise a number of wells, each one containing at least a preset antibiotic concentration to allow to assay various antibiotics at the same time.

8. A kit to assay a microorganism growth from a sample in the presence of an effective antibiotic amount comprising:

- a sterile microtitration plate, comprising different wells, each one containing an antibiotic effective amount in a stabilized state; said plate at least containing two different antibiotics in two different wells and at least one antibiotic-free well;
- a sterile container containing an adequate amount of a culture medium suitable to promote the growth of said microorganism as well as a pH indicator, preferably a red phenol.

9. A kit according to the claim 8, wherein said antibiotic is contained at least into a pair of wells, at the minimum effective concentration in the former and at a higher concentration in the latter.

10. A kit according to the claims 8 or 9, wherein said plates are prepared by dissolving the antibiotic as instructed by the Supplier, by diluting properly the obtained solution, by filling wells at the

same time with 50 µl of said solution, by using an automatic multichannel dosing device, and finally by dehydrating said wells until the solvent is full evaporated, at a temperature not able to deactivate said antibiotics.